NDI
Description

NEW DIETARY INGREDIENT NOTIFICATION INFORMATION

I. Manufacturer

Distributor of formulated dietary supplement

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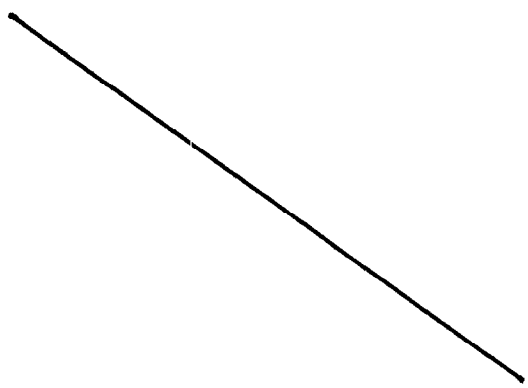
Manufacturer of *Bifidobacterium infantis* Strain 35624 biomass



II. Identification of new dietary ingredient

Name: Biomass of *Bifidobacterium infantis* Strain 35624

Bifidobacteria spp. are normal, non-pathogenic inhabitants of the human and animal gut. The name *Bifidobacterium* derives from the observation that they can exist in a Y-shaped or bifid form. They are gram positive rods that are anaerobes and have nutrient requirements that make them difficult to isolate and to grow in the laboratory. Infants are colonized by these microorganisms within days following birth. Afterwards the population of *Bifidobacteria* in the gut becomes relatively stable in normal healthy people but tends to decline with advanced age and can also be affected by diet, health status, antibiotic use, stress and possibly other factors.

B. infantis 35624 is of healthy human origin, this strain was isolated from the terminal ileum, and exhibits probiotic traits. These traits include resistance to gastric acid and bile, adherence to gut epithelial tissue, ability to persist in the gastrointestinal (GI) tract, antimicrobial substance production, immune response modulation, and *in vitro* antagonism to potentially pathogenic microorganisms or those which have been implicated in promoting inflammation.

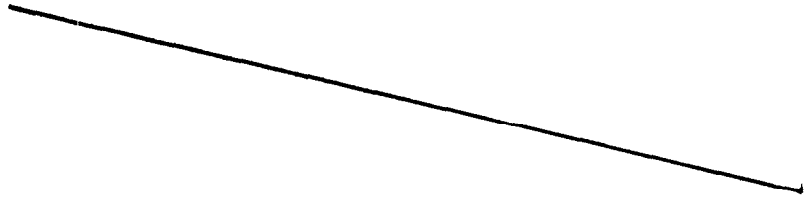
A deposit of *B. infantis* 35624 was made at the National Collection of Industrial and Marine Bacteria Limited (NCIMB) on January 13th, 1999 and given the accession number NCIMB 4103. It has not been genetically engineered or modified in any way. Further, *B. infantis* 35624 is susceptible to the following antibiotics as would be expected for *B. infantis*: ciprofloxacin, fosfomycin, gentamycin, strotomycin, tobramycin and vancomycin.

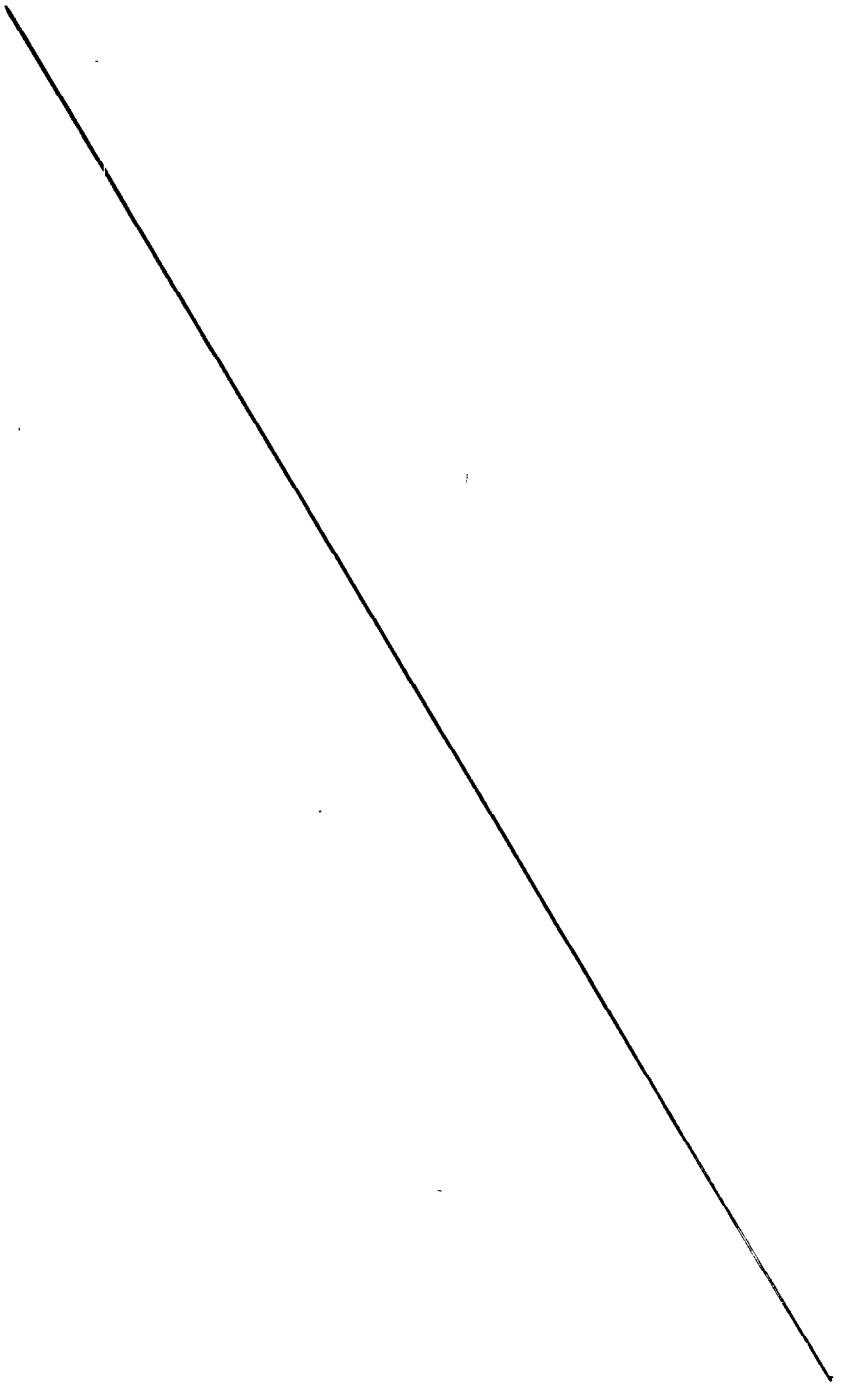
The reason for selecting a given individual strain of *B. infantis* is to have good quality control with respect to genetic drift and the possibility of contamination with other bifidobacteria. Use of this identifiable strain assures a clear linkage between studies done with the strain and the bacterial mass formulated as a dietary supplement.

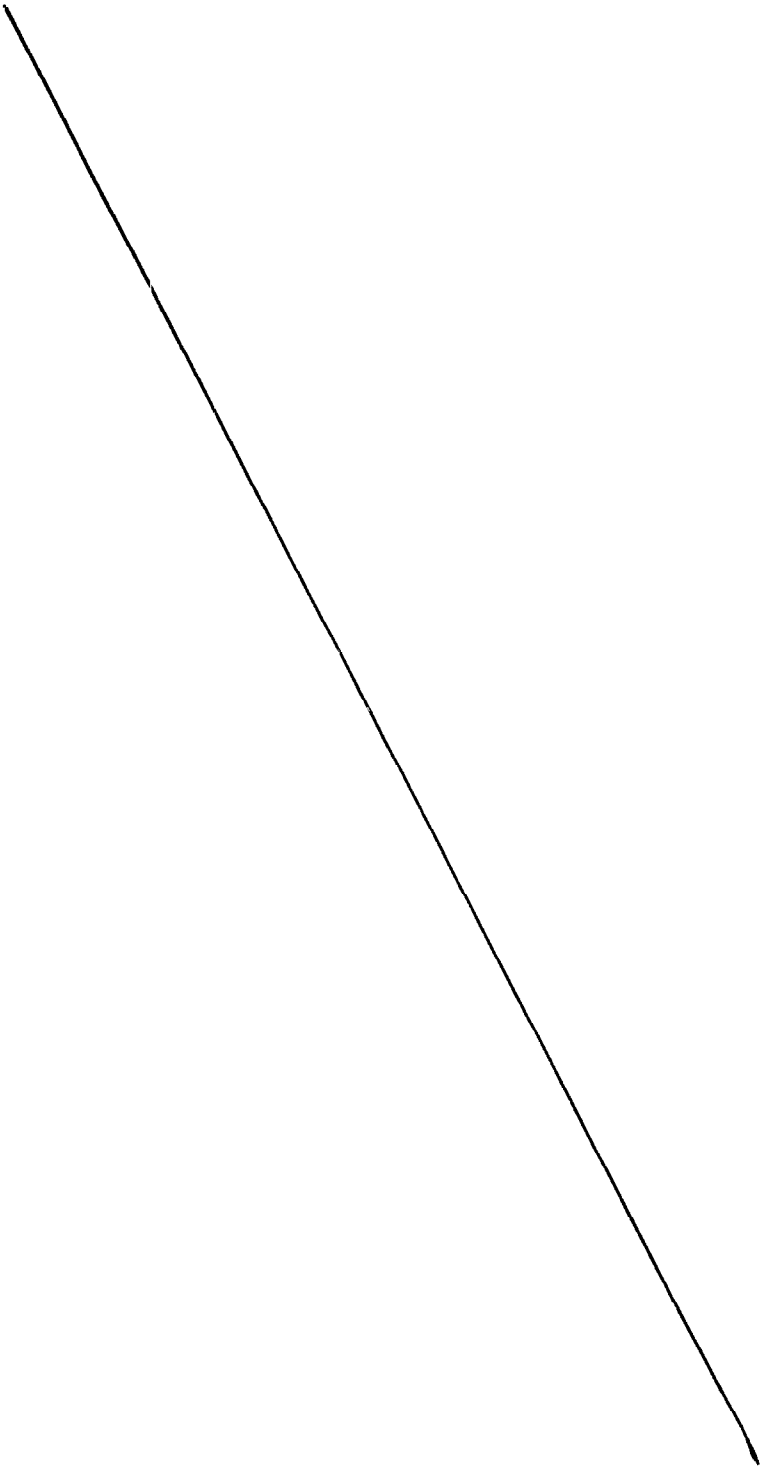
Several probiotic dietary supplements already on the market contain *B. infantis*, usually in a mixture of bacterial species. Examples include *Nature's Way Primadophilus* (total 1.5 x 10E9 cells per capsule), *MegaFlora MegaFood Probiotic Formula* (total 2 x 10E10 cells per capsule), and Wild *Oats Complete Probiotic* (total 2 x 10E9 cell per capsule).

Identification of strain using Bacterial Barcode Method

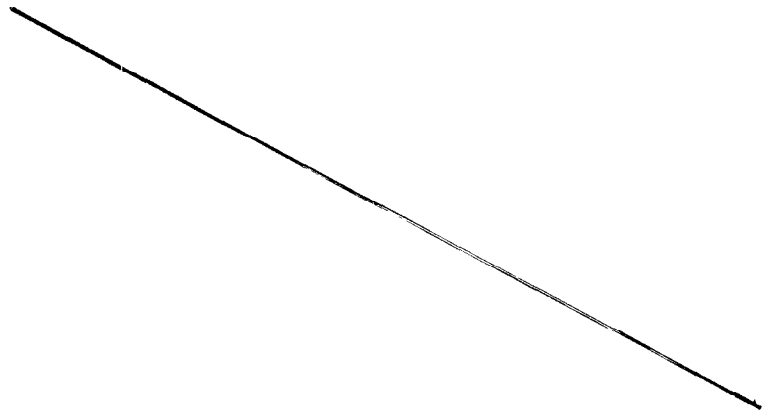
Identity of the *B. infantis* strain 35624 will be determined by Barcode analysis as described in *J Clin Microbiol.* 2005 43(1) 199-207 and *System. Applied Microbiology* 2003 26, 557-563. The method utilizes repetitive sequences which are interspersed within the bacterial genomes to give a characteristic strain specific finger-print. PCR is used to generate multiple copies (amplification) that are complementary to the interspersed repetitive sequences in the *B. infantis* 35624 genome. These are called amplicons which are analyzed by mass and charge using electrophoresis based fragment analyzer. The strain-specific rep-PCR DNA Barcode is distinct and unique to *B. infantis* 35624 strain. The name Barcode is used because of the similarity in banding patterns between the familiar barcodes used for pricing items in stores.



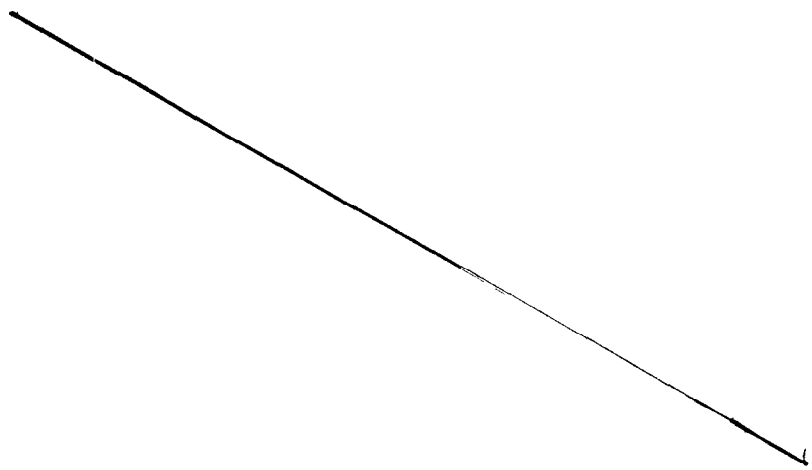




Chemical Composition of *Bifidobacterium infantis* 35624 Biomass:



The composition given above was determined from a single lot.



III. Dietary Supplement Form, Conditions of Use and Specifications

Dietary Supplement Form:

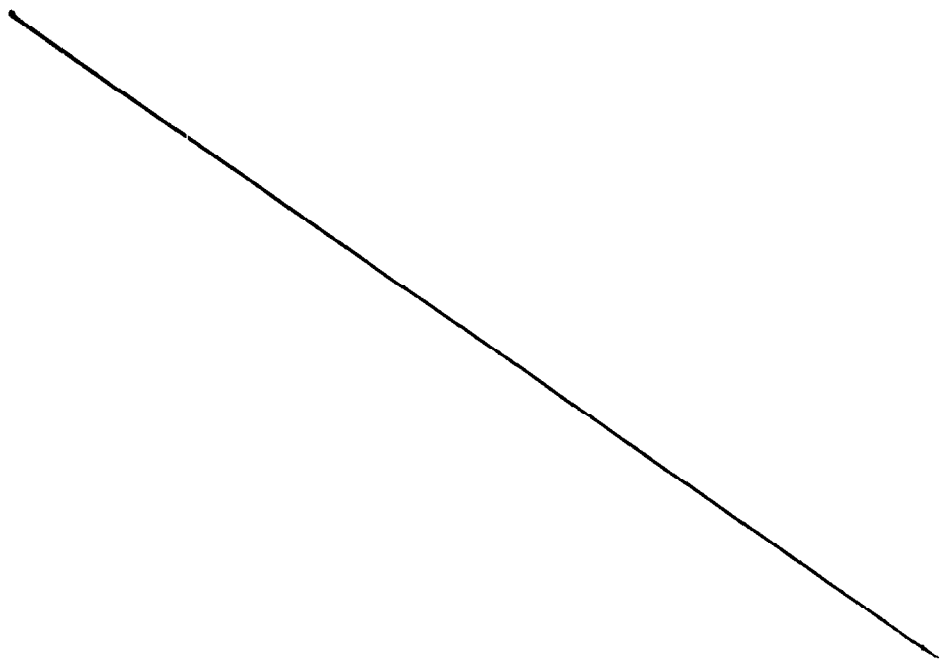
The biomass will be formulated in encapsulated form with safe and suitable ingredients, meeting food grade or pharmaceutical grade standards.

Conditions of Use:

The dietary supplement will have directions for use on the product's label to provide up to 1E+10 *Bifidobacterium infantis* 35624 per day. Bifidobacteria are naturally present in the human oral cavity and GI tract. The normal GI tract of healthy humans contain over 100 trillion bacteria of which bifidobacteria account for approximately 1%. It is also present on fresh meats and vegetables and is used in the production of cheese, yogurt, olives, sauerkraut and salami. The intended use as a probiotic as presented here is somewhat similar to the ex-US use of *Bifidobacterium* in infant formula as a probiotic for full term infants who are bottle-fed. In both cases, the intent is to increase the population of bifidobacteria within the intestinal flora.

Three *Bifidobacterium* species including *B. infantis* are listed by the Council for Responsible Nutrition (CRN) as "grandfathered" dietary ingredients under the DSHEA (CRN, 1998), and are currently being marketed as a dietary supplement in the US. Despite the acceptance of *Bifidobacterium* as an ingredient in dietary supplements, the Procter & Gamble Company wishes to have FDA's concurrence respecting safety as provided by 21 CFR §190.6.

Specifications of *Bifidobacterium infantis* 35624 biomass:



IV. Safety Assessment of the Proposed Use of *Bifidobacterium infantis* 35624

FAO/WHO Guidelines for the Evaluation of Probiotics in Food by the Working Group

The most recent and authoritative report and recommendations on data requirements for establishing the identity, benefit and safety of the use of probiotics is the report of a joint FAO/WHO Working Group on drafting guidelines for the evaluation of probiotics in food as discussed below.

The Working Group concluded that probiotic effects may be strain specific and stressed the importance of being able to link the strain to a specific beneficial health effect. We agree. While several species and strains of *Bifidobacterium* have been shown to produce beneficial effects with respect to normal functioning of the GI tract, the quality control advantages of using a specific strain far out weigh any disadvantages. Accordingly, the Procter & Gamble Company has moved forward with development of the specific strain of *Bifidobacterium infantis*, 35624, consistent with the recommendation in the guidelines. However, it is recognized that given the wide number of *Bifidobacterium* species and strains isolated from the human gut and the numerous studies performed on them, there is no basis in experience to question the safety of any of these species or strains.

The Working Group also strongly recommended that some form of DNA sequence data be used to identify and distinguish these specific strains. As indicated above this is the general means of strain identification used for *Bifidobacterium infantis* 35624. The guidelines also set forth the recommendation that the strain be deposited in an internationally recognized culture collection which has been done, a deposit of *B. infantis* 35624 was made at the National Collection of Industrial and Marine Bacterial Limited in January of 1999 with the accession number NCIMB 4103.

The report points out that historically, lactobacilli and bifidobacteria associated with food have been considered safe. Their occurrence as normal commensals of the mammalian flora and their established safe use in a diversity of foods and supplement products worldwide supports this conclusion. However, the report puts forth the caution that probiotics may theoretically be responsible for four types of side-effects:

- Systemic infections
- Deleterious metabolic activities
- Excessive immune stimulation
- Gene transfer

The Working Group acknowledges that documented correlations between systemic infections and probiotic consumption are few and have only been seen in patients with underlying medical conditions. No cases of infection from *Bifidobacterium* have been reported based on the findings in the Working Group report.

The Working Group also pointed out that the onus is on the producer to show that any given probiotic strain is not a significant risk with regard to transferable antibiotic

resistance or other opportunistic virulence properties. As *B. infantis* 35624 is sensitive to commonly used antibiotics, i.e. ciprofloxacin, fosfomycin, gentamycin, strotomycin, tobramycin and vancomycin, its use as a probiotic does not pose a risk of transferable antibiotic resistance.

For assurance of safety of bacteria used as probiotics in foods or dietary supplements, the working group recommends the following tests:

1. Determination of antibiotic resistance patterns

2. Assessment of certain metabolic activities (e.g. D-lactate production, bile salt deconjugation)

3. Epidemiological surveillance of adverse incidents in consumer (post market)

4. Assessment of infectivity of a probiotic strain in immunocompromised animals

Regarding 1, antibiotic resistance of *B. infantis 35624* has been studied and shown not to raise any questions of safety as the strain does not possess such antibiotic resistance properties.

Regarding 2, assessments of D-lactate metabolism and bile salt deconjugation are addressed below:

The probiotic bacterium *Bifidobacterium infantis* 35624 strain has been classified based upon the composition of its genome. The genome of this strain has been completely sequenced. This organism would be expected to possess the same metabolic machinery as that of other *Bifidobacterium infantis* strains. *Bifidobacterium* classically produces acetic and lactate in a molar ratio of 3:2. There is no CO_2 produced by this genus except in the case of gluconate degradation. Small amounts of formic acid, ethanol and succinic acid can also be produced by Bifidobacterium (Bergey et al 1993; De vries et al 1967). Glucose is metabolized by this genus characteristically via the fructose-6-phosphate shunt. In this pathway fructose-6-phosphate is cleaved into acetylphosphate and erythrose-4-phosphate by the enzyme fructose-6-phosphoketolase. End products of metabolism are formed by sequential action of transaldolase and transketolase, xylose-5-phophate phosphoketolase and enzymes of the Embden-Myerhof pathway action on glyceraldehyde-3-phosphate. Additional acetic and formic acid may be formed via cleavage of pyruvate. Recently it has been demonstrated that slight changes in ratio of acetate to lactate can occur in *Bifidobacterium infantis* depending on the level of oxygen present in the growth medium. Under strict anaerobic conditions the acetate/lactate ratio was 3.5:1; whereas, under oxygen conditions tolerated by this bacterium the lactate concentration increased and the acetate/lactate molar ratio was

1.5:1 (Gonzalez et al, 2004). Therefore, in the anaerobic atmosphere of the gut this organism would be expected to produce much more acetate than lactate.

D-lactate production
D-lactate has been implicated in the etiology of acidosis in children with short small bowel syndrome as well as patients with intestinal bypass (Bongaerts et al, 2000; Hove and Mortensen 1995). The production of D-lactate has been shown to be produced by various strains of the genus Lactobacillus (Kaneko et al, 1997; Iino et al, 2003). This has caused some investigators to question the use of these type probiotic strains in children (Mack, 2004). There are no reports in the literature of D-lactate production by genus *Bifidobacterium*. Furthermore, *in vitro* studies have shown that bifidobacteria supplementation can modify colonic fermentation and reduce the levels of D-lactate produced by the colonic microbial community (Jiang and Savaiano, 1997).

Assessment of bile salt deconjugation
Bifidobacteria are one of the most predominant members of the human gastrointestinal microflora. These bacteria have been utilized for several decades as probiotics due to their health-promoting beneficial effects. Probiotics or endogenous gastrointestinal tract flora must develop mechanisms to survive in the presence of significant amounts of bile salts that have detergent-like antimicrobial properties (Gunn, 2000). An important enzymatic mechanism of bile salt tolerance occurs via deconjugation of bile salts which has previously been demonstrated in *Bifidobacterium* (Grill et al, 2000; Grill et al, 1995; Tanaka et al, 20000; Tanaka et al, 1999). Bile Salt Hydrolase (BSH) is the enzyme that catalyzes the hydrolysis of glycine- and/or taurine-conjugated bile salts into amino acid residues and free bile acids. *Bifidobacterium infantis* 35624 was isolated from the gastrointestinal tract of a healthy individual. Since this organism was a member of the endogenous human gastrointestinal flora, it is expected to possess bile salt survival strategies analogous to other *Bifidobacteria infantis* strains. *In vitro* growth tests have shown that *Bifidobacterium infantis* 35624 is insensitive to bile salt at least as high as 2 % concentration in the growth medium. Recently, the BSH from *Bifidobacterium infantis* has been purified and examined in greater detail (Kim et al, 2004). This enzyme has a broad substrate range for 6 major human bile salts and has the highest activity with the glycodeoxycholic acid. The *Bifidobacterium infantis* BSH has a slight preference for glycine-conjugated bile salts over taurine-conjugated salts. It has been further speculated that BSH-activity by probiotics may be beneficial because they have the potential to reduce serum cholesterol (Anderson and Gilliland, 1999; Pereira and Gibson, 2002).

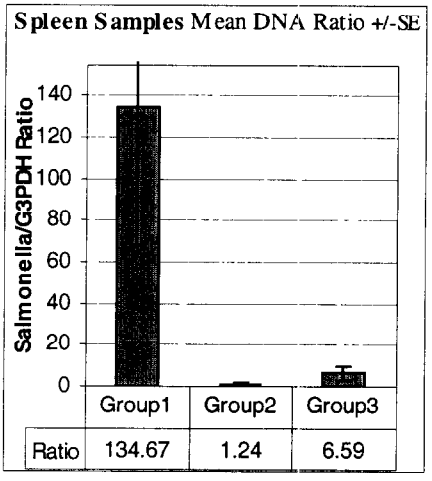
Regarding 3, post-marketing surveillance of marketed product will be conducted through follow up of consumer comments, questions and reports of alleged adverse effects. An 800 number and website will be given on the product label to facilitate feed back from consumers. Complaints, questions and comments will be collected using Procter & Gamble's electronic data capture system. Quality-related complaints will be reviewed and assessed for necessary follow-ups. Adverse event reports will

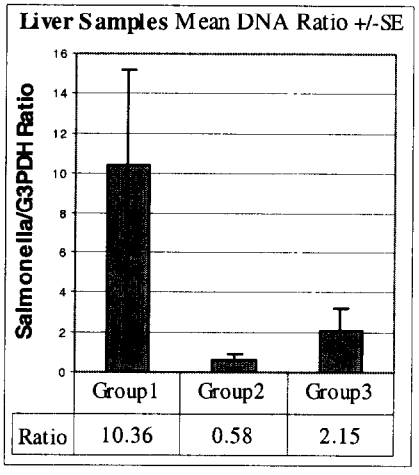
be managed by Procter & Gamble's Human Safety and Medical Affairs personnel as dictated by the nature of the report.

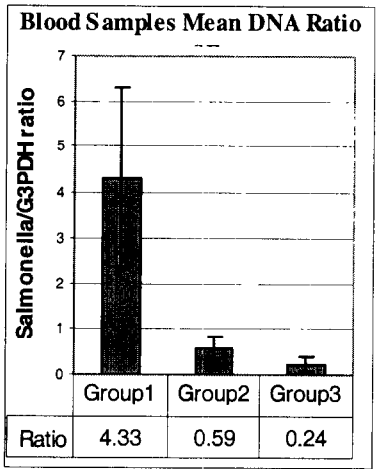
Regarding 4, the IL10 knock-out and SCID mouse models have been used to evaluate the potential benefits of administering *B. infantis* 35624 in a model for irritable bowl syndrome (IBS). Results demonstrated that the *B. infantis* 35624 strain significantly reduced disease severity (reduced weight loss, improved colon pathology, improved appearance) supporting the assertions made in other studies that *Bifidobacteria* may provide important benefits based on their activity in helping to maintain a balanced healthy intestinal flora (Dunne, et al., 1999: Dunne, 2001). O'Hara et al (2005) have shown that commensal bacteria do not induce a pathogenic response in human epithelial cells and to the contrary attenuate epithelial responses to *Salmonella typhimurium*. They investigated epithelial cell response to individual pathogenic and commensal micro-organisms as well as the modulatory effects of probiotic commensals on intestinal epithelial cell responses to pro-inflammatory stimuli. HT-29 human intestinal epithelial cells were pre-treated with or without commensal *Lactobacillus salivarius* and *Bifidobacterium infantis* for 2 h. Subsequently, the cells were infected with *S. typhimurium*. NF-κB activation in epithelial nuclear extracts was determined using the TransAM assay while IL-8 protein was measured by ELISA. In contrast to *S. typhimurium*, which increased expression of 36 genes associated with pro-inflammatory responses (e.g. TNF- •, IL-8, NF-κB), lactobacilli and bifidobacteria did not enhance expression of these genes. However, when co-cultured with *S. typhimurium*, the lactobacilli and bifidobacteria delayed NF-κB activation and reduced IL-8 production. These effects could not be attributed to interference with salmonella binding to the epithelium and occurred under both atmospheric and normoxia conditions. In contrast to their impact on salmonella-induced epithelial responses, probiotics did not offset TNF- •-induced epithelial activation. Commensal bacteria which are currently used as probiotics do not induce the well described epithelial cell response to pathogens. In contrast, they have the capacity to attenuate epithelial responses to *S. typhimurium*.

In an unpublished study conducted at Alimentary Pharmabiotic Centre, Bioscience Institute, U.C.C., Cork, Ireland, the effect of probiotic feeding on *Salmonella* translocation in a Mouse model was investigated by Sommerfield et al., (2005). The aim was to investigate the effect of probiotics on translocation in a mouse model of invasive Salmonellosis.. Thirty mature female BALB/c mice were divided into three treatment groups. Group 1, (Control group) and Group 3, (no Bif pre-feeding), were fed with skim milk solution, before infection. Group 2, (Bif pre-Fed), was fed *Bifidobacterium infantis* 35624 for 3 weeks before oral challenge. After 3 weeks, all mice were challenged with 20µl of a 10^8 CFU/ml solution of *Salmonella typhimurium* UK1 α3761. After challenge, Probiotic feeding was continued for a further six days in groups 2 & 3, with Group 3 receiving their first dose of the Bifidobacterium on the day of oral Salmonella challenge. One week after oral inoculation, the mice were sacrificed. Blood, liver and spleen samples were analyzed by molecular methods. Extraction of blood and tissue was performed using the Qiagen DNAeasy Minikit. The DNA in each sample was quantified using Molecular Probes Picogreen dsDNA

quantification assay and stored at –20°C until use. Two specific primer pairs were used to amplify and quantify either Salmonella DNA or murine housekeeping gene G3PDH, on a LightCycler, for each sample. The amount of target DNA in each sample was determined using known standards from the LightCycler data. The quantified Salmonella to G3PDH DNA ratio was calculated for each sample.
Results: There was a significant reduction ($p<0.05$) in Salmonella, detected by LightCycler, in blood, liver and spleen samples from Group 1 compared with Groups 2 and 3.







Using molecular means to detect the presence of salmonella DNA from mouse tissues and blood, it was demonstrated that Probiotic feeding reduces the amount of translocating Salmonella bacteria. Pre-feeding did not offer any additional advantage.

Brief Review of Published Studies on the Benefit and Safety of Species and Strains of *Bifidobacterium*

Bifidobacterium is a member of the lactic acid bacteria (LAB) group of microorganisms that includes *Lactobacillus*. Historically, members of this group have been consumed since humans started to use fermented milk as food. Probiotic species such as *Lactobacillus acidophilus* and *Bifidobacterium spp.* have been safely used in yogurts for more than half a century (Salminen et al., 1998). When used as probiotics, they are intended to be helpful in the maintenance of good health and the prevention of certain GI tract disorders (Naidu et al., 1999).

In 1998 the Council for Responsible Nutrition prepared a reference list of dietary ingredients "grandfathered" under DSHEA which indicates they were items of commerce prior to the enactment of DSHEA on October 15, 1994. Included on that list are *Bifidobacterium infantis*, *B. bifidium* and *B. longum*.

In 2002, an FAO/WHO Working Group drafted detailed guidelines for the evaluation and safe use of probiotics in food. They supported the conclusion of many investigators that *Bifidobacteria* associated with food use is considered safe. They

state, "Their occurrence as normal commensals of the mammalian flora and their established safe use in a diversity of foods and supplement products worldwide supports this conclusion." Further they note, "Documented correlations between systemic infections and probiotic consumption are few, and no cases of infections from *Bifidobacterium* have been reported." These guidelines have been followed and their criteria for establishing safety have been met as discussed above.

The potential benefits of orally administering *Bifidobacterium infantis* 35624 in combination with *Lb. salivarius* to an immuno-compromised mouse strain, which served as a model for irritable bowl syndrome, was examined. The results of the study demonstrated that the orally administered probiotic significantly reduced disease severity as shown by reduced weight loss, improvement in colon pathology and markedly improved appearance of the mice over a six week period. All the control mice developed a chronic wasting which was observed in the mice administered a non-probiotic dairy product (Dunne et al., 1999). Given that oral administration of *B. infantis* 35624 to immuno-compromised mice led to improvements in their health, such studies support the safety of use of *B. infantis* 35624 as a probiotic in healthy animals as well.

Human clinical trails evaluating efficacy and safety of *Bifidobacterium* were reviewed by Naidu et al. (1999). No adverse events were noted in any of the 9 studies with *Bifidobacterium* cited in Table 10 of their review (Jiang et al., 1996; Schiffrin, et al., 1995; Saavedra,et al. 1994; Langhendries, et al., 1995; Muting, et al. !968; Berrada et al. (1991); Tomoda, et al. 1991; Bennet, et al., 1992; Benno and Mitsuoka 1992). Mmost of the studies were focused on use of bifidobacteria as a probiotic or for other beneficial GI tract properties. An example of such a study is that reported by Saavedra et al., 1994 who conducted a double-blind, placebo-controlled trial in infants who were admitted to a chronic medical care hospital. The infants were randomized to receive a standard infant formula or the same formula supplemented with approximately 10^{10} *Bifidobacterium bifidum* and *S. thermophilus*. Infants were evaluated daily for occurrence of diarrhea and fecal samples, obtained weekly, were analyzed for rotavirus antigen. Fecal samples were also obtained during episodes of diarrhea for virological and bacteriological analysis. 31% of the placebo-controls developed diarrhea while only 7% of the probiotic treated infants developed the condition, $p = 0.035$ using Fisher's two tailed, exact test. 39% of the controls and 10% of the treated infants shed rotavirus at some time during the study, $p = 0.025$. The authors concluded that supplementation of infant formula with 10^{10} *B. bifidum* and *S. thermophilus* can reduce the incidence of acute diarrhea and rotavirus shedding in infants admitted to the hospital.

A recent study by Gopal et al. (2003) addressed the desirability and benefit of an increase in the number and activity of bifidobacteria and lactobacilli in the colon and demonstrated that oral administration of live, beneficial microbes can achieve this result. Thirty subjects between the ages of 20 and 60 years were randomly assigned to three groups. Subjects in group 2 received 3×10^{10} CFU of *Bifidobacterium lactis* in supplemented milk per day for four weeks. Fecal sample were collected every week

and analyzed for eight major groups of microbes associated with the human GI tract. Subjects receiving bifidobacteria exhibited a significant increase in the fecal counts of both lactobacilli ($p< 0.004$ and bifidobacteria ($p<0.0002$). Little or no changes were seen in subjects who consumed milk without supplementation. Bifidobacteria was clearly demonstrated to have survived passage through the human GI tract.

While an extensive literature of clinical studies on Lactobacillus and *Bifidobacterium* supports the safe use of this LAB group of microorganisms as probiotics in humans, the studies are focused primarily on the beneficial effects of *Bifidobacterium*, not on safety *per se*. The reasonable conclusion that can be drawn from these studies on beneficial effects is that they demonstrate *Bifidobacterium*'s effectiveness in restoring a healthy state without side effects which speaks to its safety. For example, the finding that *Bifidobacterium* improves irritable bowl syndrome or other adverse conditions of the GI tract in humans or animals is evidence that the organism is not harmful to the GI tract but is instead healthy and therefore safe. As *Bifidobacterium* has never been shown to lead to systemic infections in humans, such beneficial effects in the GI tract support the safety of the organism for its use as a probiotic.

Also providing support for the safety of bifidobacteria is their natural presence in the normal, healthy human gut. Bifidobacteria constitute a major part of the normal intestinal microflora in humans throughout life. They appear in the stools a few days after birth and increase in number thereafter. The number of bifidobacteria in the colon of adults is $10^8 - 10^{11}$ CFU/g but this number decreases with age (Orrhage and Nord, 2000).

Review of Published Clinical Study of the Benefit and Safety of *Bifidobacterium infantis* 35624

O'Mahoney et al. (2005) have investigated the efficacy of B. infantis 35624 in randomized, placebo-controlled, double-blind comparison of the probiotic bacteria lactobacillus and *Bifidobacterium* in irritable bowel syndrome (IBS).
The primary aim of the study was to compare the response of symptoms and cytokine ratios in patients with irritable bowel syndrome to ingestion of milk-based probiotic preparations containing a well-characterised *Lactobacillus* or *Bifidobacterium* strain.

Seventy-seven subjects with irritable bowel syndrome diagnosed according to Rome II criteria were randomized to receive either *L. salivarius* UCC4331 or *B. infantis* 35624, each in a dose of $1x10^{10}$ live bacterial cells in a malted milk drink, or the malted milk drink alone as placebo for 8 weeks. The cardinal symptoms of irritable bowel syndrome were recorded on a daily basis and assessed each week. Quality of life assessment, stool microbiological studies and blood sampling for estimation of peripheral blood mononuclear cell release of the cytokines IL-10 and IL-12 were performed at the beginning and at the end of the treatment phase.

For all symptoms, with exceptions of bowel movement frequency and consistency, the group randomised to *B. infantis* 35624 experienced a greater reduction in symptom scores during the treatment period; composite scores, as well as individual scores for abdominal pain/discomfort, bloating/distension and bowel movement difficulty, were significantly lower than placebo for those randomized to the B*ifidobacterium* for most weeks of the treatment phase. At baseline, irritable bowel patients demonstrated an abnormal IL-10:Il-12 ratio, indicative of a pro-inflammatory, Th-1 state. This ratio was normalized by B*ifidobacterium* feeding alone.

Bifidobacterium infantis 35624 alleviated symptoms in irritable bowel syndrome. This symptomatic response was associated with a normalization of the ratio of an anti-inflammatory to a pro-inflammatory cytokine, suggesting an immune-modulating role for this organism, in this disorder.

Furthermore the authors stated that treatment with *B. infantis* 35624 was well tolerated and free of significant adverse events.

V. Summary and Conclusion

Bifidobacterium is present in the human gut shortly after birth. Approximately, one trillion of the live organisms in the human gut belong to the *Bifidobacterium* genus. Human foods, particularly fermentation products like yogurts and cheeses, have large numbers of bifidobacteria. *Bifidobacterium* is used worldwide as a probiotic including use in infant formula for bottle-fed infants. Despite this widespread use there have been no reports of systemic infections. Numerous studies have been conducted on different *Bifidobacteria* strains and species without any indication of harmful effects. The subject, *Bifidobacterium infantis* 35624, has been subjected to the safety criteria proposed by the FAO/WHO Working Group that developed guidelines for the evaluation of probiotics in food and dietary supplements. These safety criteria have been met and satisfied by the studies and analyses described herein. Additionally, a published clinical study confirms both the benefit and safety of *Bifidobacterium infantis* 35624 when used to treat irritable bowl syndrome. Based on these findings it is concluded that the intended use of *Bifidobacterium infantis* 35624 will reasonably be expected to be safe in accordance with the safety standard set forth in 21 CFR §190.6.

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